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Gene transcription profiles of *Saccharomyces cerevisiae* after treatment with plant protection fungicides that inhibit ergosterol biosynthesis ☆

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Abstract

Resistance to agricultural fungicides in the field has created a need for discovering fungicides with new modes of action. DNA microarrays, because they provide information on expression of many genes simultaneously, could help to identify the modes of action. To begin an expression pattern database for agricultural fungicides, transcriptional patterns of *Saccharomyces cerevisiae* strain S288C genes were analyzed following 2-h treatments with I_{50} concentrations of ergosterol biosynthesis inhibitors commonly used against plant pathogenic fungi. Eight fungicides, representing three classes of ergosterol biosynthesis inhibitors, were tested. To compare gene expression in response to a fungicide with a completely different mode of action, a putative methionine biosynthesis inhibitor (MBI) was also tested. Expression patterns of ergosterol biosynthetic genes supported the roles of Class I and Class II inhibitors in affecting ergosterol biosynthesis, confirmed that the putative MBI did not affect ergosterol biosynthesis, and strongly suggested that in yeast, the Class III inhibitor did not affect ergosterol biosynthesis. The MBI affected transcription of three genes involved in methionine metabolism, whereas there were essentially no effects of ergosterol synthesis inhibitors on methionine metabolism genes. There were no consistent patterns in other up- or downregulated genes between fungicides. These results suggest that inspection of gene response patterns within a given pathway may serve as a useful first step in identifying possible modes of action of fungicides.
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☆ The microarray data in this manuscript have been deposited in the NCBI Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/projects/geol/), under acession number GSE2412.

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1. Introduction

Inhibition of ergosterol biosynthesis is a common target of agricultural fungicides used for plant protection. It is generally an effective target because sterols are essential components of eukaryotic cells [1]. Ergosterol, the main sterol of most fungi [2,3], is an important part of fungal membranes. Inhibition of ergosterol biosynthesis affects membrane fluidity [4] and permeability [5,6]. In *Saccharomyces cerevisiae* (baker's yeast), ergosterol is also a major component of secretory vesicles [7] and has an important role in mitochondrial ATPase activity [8]. Ergosterol is essential for yeast growth, although growth can occur in the presence of other sterols if a small "sparking" amount of ergosterol is present [9,10].

The inhibitors affecting the part of the isoprenoid pathway committed to ergosterol biosynthesis are quite selective; consequently, they do not affect biosynthesis of cholesterol, the main animal sterol, or of sitosterol, the principal plant sterol [2]. Sterol biosynthesis inhibitor (SBI)[4] fungicides, or ergosterol biosynthesis inhibitor fungicides, are used in both medicine and agriculture. Considerable information exists on the physiological and biochemical effects of SBIs, although less is known of their effects on fungal gene expression. SBIs fall into four classes [11]. All four classes inhibit enzymes for the synthesis of intermediates between squalene, the first isoprenoid intermediate committed to sterol biosynthesis, and ergosterol, the end product (Table 1). Class I inhibitors consist of the demethylation inhibitors (DMIs) that inhibit lanosterol C-14 demethylase, responsible for the C14-demethylation step of sterol biosynthesis [1,12]. This demethylase is encoded by the *ERG11* gene of *S. cerevisiae*. DMIs include five chemical classes: triazoles, imidazoles, piperazines, pyridines, and pyrimidines, with the greatest number of fungicides in the triazole group [11]. Class II inhibitors consist of the morpholine-type compounds, which inhibit to varying degrees the C14-reductase and the Δ8–Δ7-isomerase of ergosterol biosynthesis. Those enzymes are encoded by *ERG24* and *ERG2* of yeast, respectively. Morpholine compounds include morpholines, piperadines, and spiroketolamines. Class III inhibitors interfere with the sterol C-3 keto reductase involved in the conversion of 4,4-dimethylzymosterol into zymosterol [1] and encoded by *ERG27* [13]. The one known inhibitor in this class is fenhexamid, a hydroxyanilide [11]. Class IV inhibitors, which consist of thiocarbamates and allylamines, interfere with squalene epoxidase, which is encoded by *ERG1* and responsible for the first committed step of sterol biosynthesis (oxidation of squalene to 2,3-oxidosqualene) [1,2,11]. This last class contains medicinal fungicides, but no agricultural fungicides [14].

In both medicine and agriculture, widespread use of *ERG11* inhibitors has led to evolution of resistance [12,15,16]. Mechanisms of resistance that have been discovered include mutations in ATP binding cassette (ABC) transporters [17], high expression levels of multidrug resistance genes [18], multiple copies of *ERG11* transcriptional enhancers [19], high rates of fungicide efflux from cells [20], and mutation or overexpression of the *ERG11* gene [12,16,18].

Detection of the global expression response of the fungal genome after treatment with fungicides is possible with DNA microarrays. Such information may lead to detection of other genes that are mutated or overexpressed in resistant fungi, thus providing more information on mechanisms by which resistance evolves [12]. No whole-genome DNA microarrays existed for plant pathogenic fungi when this study was begun, and so *S. cerevisiae* was used as a model organism to study fungal gene expression in response to agricultural fungicides.[5] Microarrays of *S. cerevisiae* are readily

[4] *Abbreviations used:* SBI, sterol biosynthesis inhibitor; DMI, demethylase inhibitor; TIGR, the Institute for Genomic Research; MBI, methionine biosynthesis inhibitor; DMSO, dimethylsulfoxide.

[5] A whole-genome microarray for *Magnaporthe grisea*, the rice blast fungus, is now commercially available from Agilent (http://www.chem.agilent.com/Scripts/PDS.asp?lPage=9894).

Table 1
Genes and corresponding enzymes of ergosterol biosynthesis, in order of occurrence in the biosynthetic pathway

Gene	Enzyme encoded	Targeting SBI
ERG10	Acetoacetyl-CoA thiolase	
ERG13	HMG-CoA synthase	
HMG1, *HMG2*	HMG-CoA reductase	
ERG12	Mevalonate kinase	
ERG8	Phosphomevalonate kinase	
ERG19	Mevalonate pyrophosphate decarboxylase	
IDI1	Isopentenyl diphosphate isomerase	
ERG20	Geranyl pyrophosphate synthase	
ERG9	Squalene synthase	
ERG1	Squalene epoxidase	Class IV SBI (medical fungicides)
ERG7	Lanosterol synthase	
ERG11	Lanosterol C-14 demethylase	Class I SBI
ERG24	Sterol C-14 reductase	Class II SBI
ERG25	Sterol C-4 methyloxidase	
ERG26	Sterol C-3 dehydrogenase	
ERG27	Sterol C-3 keto reductase	Class III SBI
ERG28	Unknown function	
ERG6	Sterol C-24 methyltransferase	
ERG2	Sterol C-8 isomerase	Class II SBI
ERG3	Sterol C-5 desaturase	
ERG5	Sterol C-22 desaturase	
ERG4	Sterol C-24 reductase	

available, and this fungus has been used as a model organism for gene expression studies with pharmaceutical fungicides, including those that inhibit ergosterol biosynthesis [12,21,22]. Others have used DNA microarrays to study resistance mechanisms to ergosterol synthesis inhibitors in *Candida albicans* [23,24] and mechanism of action of the cell wall synthesis inhibitor caspofungin in *S. cerevisiae* [25]. Parveen et al. [26] recently found evidence that α-terpinene inhibits ergosterol synthesis in *S. cerevisiae* through interpretation of microarray results. In the agricultural realm, yeast microarrays have been used to study gene expression in response to the herbicide sulfometuron methyl, an inhibitor of branched-chain amino acid biosynthesis [27].

Besides aiding in identification of new mechanisms of resistance, the gene expression profiles obtained from microarrays can be used to identify a particular mode of fungicide action. Studies of gene expression profiles of yeast treated with pharmaceutical ergosterol biosynthesis inhibitors have led to identification of a subset of genes that are up- or down-regulated in response to these compounds, and to the determination of the mode of action of an unknown compound based on the similarity of its gene expression profile to those of the ergosterol biosynthesis inhibitors [12]. Other yeast microarray studies with clinical immunosuppressants have indicated that gene expression patterns in response to those treatments provide a reliable picture of the pathways affected [28]. A more recent study of agricultural fungicides indicates that microarrays help in grouping fungicides by structural similarity and suggests that gene expression profiles can provide information on fungicide toxicity [29].

As fungicides with different molecular target sites are likely to differ in some of the genes they affect, development of a library of gene expression profiles for agricultural fungicides with different molecular target sites should be possible. Such a library might be used as a first step to identify molecular target sites, providing a snapshot of pathways that may be affected and a list of genes whose expression would be worth confirming by

real-time PCR. To begin building this library, we have examined the gene expression profiles of eight agricultural SBI fungicides. Class I, Class II, and Class III inhibitors are represented, including representatives of several chemical groups within the large family of Class I inhibitors. For comparison, we have also examined a non-SBI, cyprodinil, a putative inhibitor of methionine synthesis [30–33]. Expression patterns of ergosterol and methionine biosynthetic genes in response to these nine treatments indicate that focusing on metabolic pathways is a useful first step in identifying genes worth examining more closely in in-depth mode-of-action studies.

2. Materials and methods

2.1. Yeast strain, media, and culture conditions

Stocks of the haploid yeast strain S288C were stored in 25% (v/v) glycerol at −80 °C. Cultures were initiated from single colonies obtained by streaking a small aliquot of a glycerol stock onto YPD (yeast peptone dextrose) agar (50 g/L Difco YPD media, #242820;15 g/L agar) and incubating the plate at 30 °C for 48 h. Plates of single colonies, used to initiate liquid cultures, were stored at 4 °C for 1–4 weeks. For I_{50} determinations and treatments for microarray experiments, yeast were grown in filter-sterilized synthetic dextrose medium (SDM), consisting of 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate (Difco #0919-07), 5 g/L ammonium sulfate, 20 g/L glucose, and 165 mM MOPS. The pH was adjusted to 7.0 with NaOH. Flask cultures were grown at 30 °C and shaken at 250 rpm in an Innova 4230 Refrigerated Incubator Shaker (New Brunswick Scientific, NJ).

2.2. Reagents and chemicals

Of the Class I inhibitors, two imidazoles (imazalil and prochloraz), one pyrimidine (fenarimol), and two triazoles (fenbuconazole and triadimefon) were studied. Class II inhibitors consisted of the morpholines dodemorph and fenpropimorph, and Class III was represented by fenhexamid. Technical grade fungicides were obtained from the following sources: Fenpropimorph was from Riedel-de-Haën (Milwaukee, WI); and fenarimol, fenbuconazole, imazalil, prochloraz, triadimefon, cyprodinil, dodemorph, and fenhexamid were from ChemService (West Chester, PA). Molecular biology grade dimethylsulfoxide (DMSO), from Fisher, and absolute ethanol were used. All other chemicals were of reagent grade or better.

2.3. Determination of I_{50} values of the different fungicides/antifungal compounds

The I_{50} concentration of each fungicide was used for studying gene expression changes, as 50% inhibition should cause enough changes in gene expression to understand the processes affected by the fungicide, without causing too many gene expression changes due to secondary effects that might occur at higher doses [21]. Also, since Jia et al. [27] found that going from I_{40} to I_{98} concentrations of sulfometuron methyl did not greatly change the gene expression profile, I_{50} doses of fungicides seemed likely to give a good profile of fungicide effects. To determine I_{50} values, an overnight yeast culture was prepared by inoculating 100 mL SDM in a 500-mL Erlenmeyer flask with a single yeast colony and growing the culture for 16–24 h. The culture was diluted in SDM to $A_{600} = 0.10$ and then used to start 100-mL cultures in 250-mL Erlenmeyer flasks. These were allowed to grow to $A_{600} = 0.2$ (the beginning of logarithmic growth), at which time they were treated with a range of concentrations of the fungicide, which were prepared in duplicate and dissolved in DMSO or ethanol (final concentration 0.5%). Controls received equivalent volumes of solvent. Eighteen to twenty hours later, the A_{600} of the cultures was measured, and the concentration causing 50% growth inhibition relative to the control (I_{50}) was determined graphically. The experiment was generally repeated with a smaller range of duplicate concentrations to target more precisely the I_{50}, which was the concentration then used to treat yeast cultures grown for microarray studies.

2.4. Growth and harvest of yeast cultures for microarray studies

Most experiments consisted of two biological replicates performed on two different days. A few fungicide treatments were done more than twice to confirm results, namely, imazalil ($N = 4$), triadimefon ($N = 3$), and fenhexamid ($N = 3$). Cultures were started from a diluted overnight culture as described above, and the previously determined I_{50} concentration of fungicide was added at $A_{600} = 0.2$. Controls received equivalent amounts of DMSO (final concentration 0.5%). After about 2 h (A_{600} of about 0.4 for the control), the cultures were harvested. Duplicates of the harvested cultures were allowed to grow for an additional 16–18 h, to determine if the fungicide concentrations used had indeed caused 50% growth inhibition. Growth was determined as a percentage of the control (set at 100%). Harvested cells were used for microarray experiments if the growth in the duplicate flasks 18–20 h after treatment was 40–60% of the control.

2.5. Cell harvesting and RNA preparation

Yeast cells were transferred to 50-mL polypropylene Falcon tubes and centrifuged at 3000*g* at room temperature for 5 min. The supernatant was discarded; tubes were centrifuged again for 1 min at 3000*g*, and the remaining supernatant was removed. Tubes were flash-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNA was isolated as described by Agarwal et al. [21], and mRNA was isolated with the Qiagen Oligotex mRNA kit. Both total RNA and mRNA were quantified with a Pharmacia GeneQuantII RNA/DNA Calculator.

2.6. Preparation and labeling of cDNA

Synthesis of cDNA from mRNA, and subsequent labeling of cDNA with Cy3 and Cy5 dyes, were done according to a protocol from The Institute for Genomic Research (TIGR) [34]. The dyes used to label the control and the fungicide-treated sample were switched (dye swap) between biological replicates to minimize biasing of data by differences in dye labeling efficiency [27]. The following changes to the TIGR protocol were made: the amount of mRNA used for the cDNA synthesis was decreased to 1 μg; 2.5 μg of oligo(dT)$_{12–18}$ primer (Invitrogen #18418-012) was used instead of random hexamers to prime cDNA synthesis; and volumes of reagents for this part of the protocol were increased by 33%, except for the volume of reverse transcriptase, which was increased to 220 U. In addition, the final concentration of aminoallyl-dUTP used for cDNA synthesis was increased to 300 μM, while the concentration of dTTP was decreased to 200 μM. After hydrolysis of RNA from cDNA products, reactions were neutralized with 25 μL 1 M HEPES, pH 7.0. Subsequent purification was done with the Qiagen MinElute Reaction Cleanup kit (#28204) instead of the Qiagen PCR Purification kit (#28004), as the binding buffer in the former contained a pH-sensitive dye facilitating recognition of insufficiently neutralized reactions. Reaction mixtures were mixed with 3.5 volumes of binding buffer and 3.5 μL 3 M sodium acetate, pH 5.2, before being transferred to columns. The purified cDNA was eluted in 20 instead of 60 μL of buffer. Dye coupling reactions were quenched with 250 mM instead of 100 mM sodium acetate, and labeled cDNA was eluted in 80 instead of 60 μL of buffer.

Labeling efficiency was determined by measuring absorbances of the entire sample in a quartz microcuvette, as recommended by the TIGR protocol. Absorbances at 260 nm (cDNA), 550 nm (Cy3), and 650 nm (Cy5) were measured on a Shimadzu UV-3101PC spectrophotometer. Frequency of incorporation (FOI, no. labeled nucleotides per 1000 nucleotides) was calculated according to the Corning GAPS II Coated Slides Instruction Manual, pp. 6–7 (avail. online from www.corning.com/lifesciences), using the equation

$$\mathrm{FOI} = (\text{pmol dye incorporated} \times 324.5)/\text{ng cDNA},$$

where pmol Cy3 $= A_{550}/0.15$ and pmol Cy5 $= A_{650}/0.25$.

Total incorporated Cy dye ranged from 25 to 326.3 pmol, and the FOI was generally between 10 and 20.

2.7. Hybridization and image processing

Saccharomyces cerevisiae microarrays from the Ontario Cancer Institute were used. These contained all identified 6400 open reading frames (ORFs), in duplicate. The Cy-labeled sample, with its corresponding Cy-labeled control, were combined and dried in a vacuum centrifuge (SpeedVac, Eppendorf). The residue was resuspended in 120 μL hybridization solution (41% formamide, 41% 5× SSC, 16% SDS, 1.6% 1 mM DTT, filter sterilized through a 0.22 μm syringe filter) with 0.01% (w/v) sheared salmon sperm DNA (Gibco-BRL, #15632-011) as a blocking agent. The probe in hybridization solution was denatured by heating at 95 °C for 5 min, centrifuged at 12,000*g* for 2 min to precipitate debris, and introduced onto a microarray at 42 °C in a GeneTAC hybridization station (Genomic Solutions, Ann Arbor, MI). The microarray was incubated with agitation of the probe at 42 °C for 16 h, followed by three washes of increasing stringency. Each wash step was done twice and consisted of a 10-s flow and 20-s hold of wash buffer. Wash solutions were those recommended for post-hybridization washing in the Corning GAPS II manual, with the final two washes at 25 °C. Slides were then removed from the hybridization station, dipped once in 0.1× SSC, and dried with compressed air. Arrays were scanned on a ScanArray 5000 confocal laser scanner (Packard Bioscience) with 10 μm resolution using the ScanArray 3.0 software [35]. Each slide was scanned at 550 nm to measure intensity of hybridization to Cy3-coupled cDNA, and at 650 nm to measure intensity of hybridization to Cy5-coupled cDNA. Data for the two scans were collected in Cy3 and Cy5 channels and stored as separate TIFF images.

2.8. Microarray data analysis

Spots were identified and their intensities measured in the QuantArray 3.0 software (Packard Bioscience) [36]. Background subtraction and LOWESS sub-grid normalization were done with the GeneTraffic Multi software package from Iobion Informatics (La Jolla, CA). These calculations were done for both the Cy3 and Cy5 scans. The ratio of the normalized, background-subtracted intensity of the treatment to the normalized, background-subtracted intensity of the control was determined for each gene. Annotation of genes was based on the Comprehensive Yeast Genome Database (http://mips.gsf.de/genre/proj/yeast/index.jsp) and the *Saccharomyces* Genome Database (www.yeastgenome.org).

Yeast microarray experiments sometimes contain only one or two replicates because of the high cost of the microarrays. With fewer than three replicates, standard deviations of means cannot be calculated. Consequently, genes are sometimes considered significantly up- or downregulated relative to the control if expression levels are above or below certain treatment-to-control ratios (2.5-fold increase/decrease used by De Backer et al. [24]; 2-fold increase or decrease in both replicates used by Agarwal et al. [21]; 1.5-fold increase or decrease in both replicates used by Zhang et al. [22]. In this study, a 2-fold increase or decrease in all replicates of a treatment (2–4) was considered an indicator of a significant change in gene expression when looking at effects of separate fungicides. Also, since all fungicides within a given SBI class inhibit the same enzyme(s) of ergosterol biosynthesis, the effects of Class I, Class II, and Class III SBIs on enzymes of the ergosterol biosynthetic pathway were compared by taking the average fold change in intensity for the duplicate spots on a chip and calculating the mean intensity for all biological replicates of a fungicide treatment, then pooling means from all fungicides within a class (five means for Class I and two for Class II). Standard errors of those pooled means were then calculated. Because only one fungicide was examined within Class III SBIs and the MBI class, means and standard deviations were calculated for the biological replicates within those classes.

The same method of analysis was used to evaluate the effects of the three classes of SBIs on methionine biosynthetic genes, cell cycle genes and branched-chain amino acid biosynthetic genes. Other genes were similarly analyzed if upregulated 2-fold or more in each of the duplicate results on a microarray, for at least two biological replicates of a treatment, and among at least 80% of the fungicides within a class. In cases where

gene expression levels were close to the 2-fold cutoff (200–300% of the control), genes were not considered significantly upregulated if the standard deviation exceeded 20% of the mean. Genes that were up- or downregulated 2-fold or more, but whose intensity readings (before or after background subtraction) included values below 200, were excluded from analysis because these values were considered outside of the sensitivity range of the scanner.

A couple of modifications was made to the data when calculating mean fold change within a class. Fold changes greater than 10 were listed as 10, since higher values would probably be inaccurate, given the sensitivity limitations of the scanner. Also, the GeneTraffic method of presenting fold change is to divide the intensity reading of the treatment by the normalized intensity of the control. If the intensity of the treatment is less than that of the control, the inverse of the treatment:control ratio is used and given a negative sign (GeneTraffic DUO User Manual, version 2.8, 2003). To avoid negative ratios, the ratio of the treatment to the control was calculated for all genes, and changes were given as a percentage of the control. Consequently, significantly downregulated genes had treatment:control intensity ratios that were 50% or less of the control, and significantly upregulated genes had treatment:control intensity ratios that were 200% or more of the control.

3. Results and discussion

3.1. Dose–response studies

The I_{50} concentrations obtained in dose–response studies were used to determine the effects of fungicide treatments on gene expression. The toxicity of the nine fungicides to yeast varied considerably after 18–20 h of treatment, with I_{50} values ranging from 12.5 μM for fenbuconazole, to 350 μM for triadimefon (Fig. 1). The shapes of dose-response curves differed in some experiments, as the highest fungicide concentrations did not always completely inhibit growth. Because the goal of the dose-response studies was to determine the I_{50} and not the MIC (minimum inhibitory concentration needed for zero growth), experiments were not repeated with higher fungicide concentrations when the MIC was not reached. It is possible that complete inhibition of growth would not have occurred if higher concentrations had been attempted, as some fungicides precipitated when highly concentrated DMSO solutions were added to media. Also, some of the compounds may have been fungistatic instead of fungitoxic. Furthermore, slow uptake of some of the compounds could have resulted in some growth occurring before the compound reached its molecular target site. Other fungicide treatments yielded dose-response curves whose slopes changed dramatically over the range of concentrations tested, with a very steep slope around the I_{50} value. The I_{50} values of these fungicides were the most variable, and in cell-harvesting experiments, achieving 50% growth sometimes required inoculating inoculate cultures with several concentrations bracketing the I_{50} value that had been determined in dose–response curves.

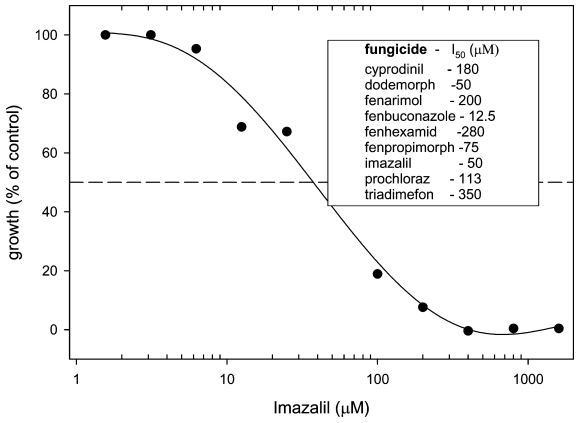

Fig. 1. Example of a dose–response curve (with imazalil) used to determine fungicide I_{50} values provided in the inset. I_{50} values were determined 18–20 h after inoculation of cultures with fungicides.

3.2. Effects of fungicide treatments on ergosterol biosynthetic genes

The difficulty of analyzing information and identifying false positives from a set of 6400 genes (about 51,200 data for eight SBI treatments) led to analyzing data subsets containing genes likely to be affected by the treatments. Fig. 2 depicts the

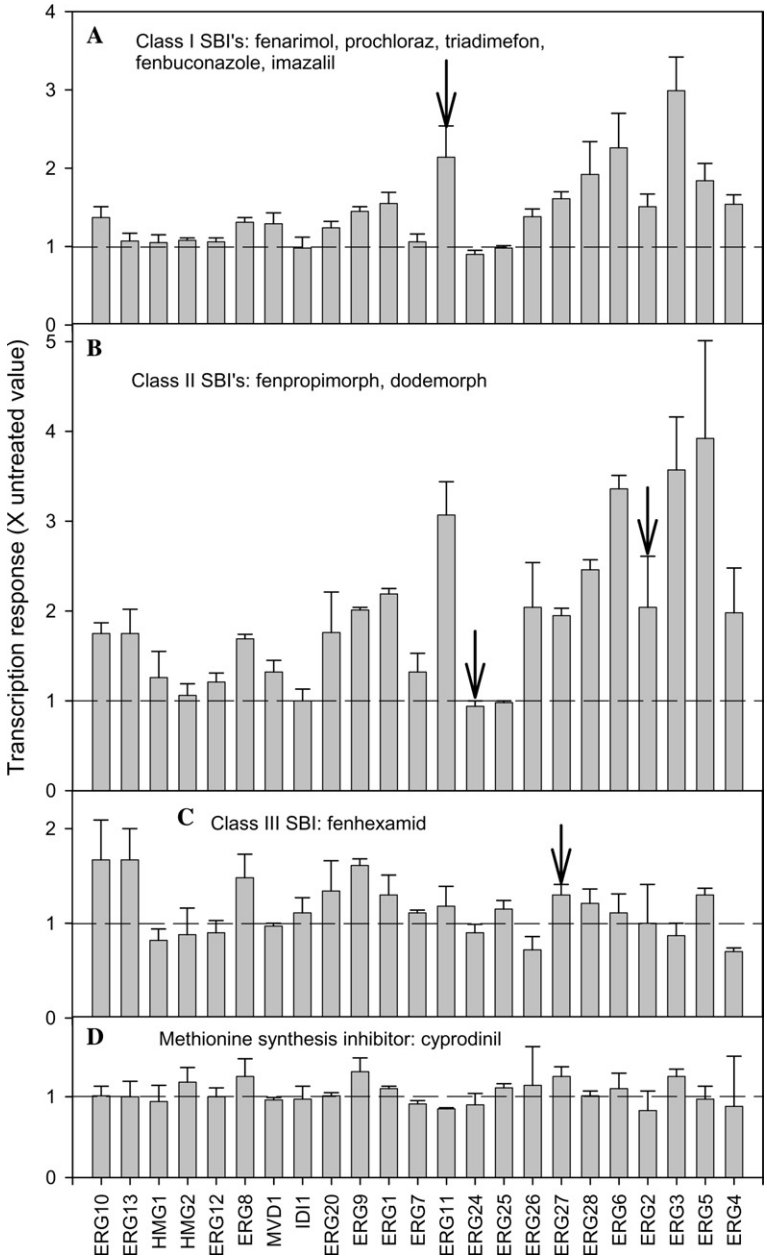

Fig. 2. Effects of Class I (graph A), II (graph B), and III (graph C) sterol biosynthesis inhibitors, and a putative methionine biosynthesis inhibitor (cyprodinil, graph D) on expression levels of genes in the ergosterol pathway. Standard errors are shown on graphs A and B, and standard deviations are shown on graphs C and D. Genes are listed on the *x*-axis from left to right in the order in which they appear in the pathway. The transcription relative to untreated controls (referred to in the text as a percentage of the control) is shown on the *y*-axis. Dashed horizontal lines on the graphs indicate the level of expression at which no change is seen relative to the control. Arrows indicate gene(s) targeted by the inhibitor.

effects of cyprodinil, a putative MBI, and the three classes of SBIs on ergosterol biosynthetic genes, with genes on the *x*-axis in the order by which biosynthesis proceeds from acetyl-CoA (the starting molecule in the cytosolic isoprenoid pathway) to ergosterol. Inspection of Fig. 2 reveals similar gene expression patterns in cells treated with Class I and Class II inhibitors. Both classes caused a general increase in gene expression in the latter part of the ergosterol pathway, from *ERG11* to *ERG4*. Treatment with Class I inhibitors caused an increase of more than 200% of the control in expression of *ERG11* ($214 \pm 41\%$), *ERG6* ($226 \pm 44\%$), and *ERG3* ($299 \pm 43\%$). Expression of those three genes in response to pharmaceutical Class I SBIs was observed in microarray studies with *C. albicans* [24] and *S. cerevisiae* [12,21], although other *ERG* genes upregulated in those studies were not responsive in this study. The upregulation of *ERG11* and other *ERG* genes is thought to be a general response to decreased ergosterol levels [24,37]. *ERG11* expression in *C. albicans* has been shown to increase in the presence of fenpropimorph, again suggesting that *ERG11* expression increases as a general response to decreased ergosterol levels, regardless of the targeted enzyme [37].

In agreement with the above-mentioned results on general upregulation of *ERG* genes, we found that Class II inhibitors also stimulated expression of *ERG11*, *ERG6*, and *ERG3*, as well as expression of *ERG9*, *ERG1*, *ERG26*, *ERG28*, *ERG2*, and *ERG5* (Fig. 2B). The lack of change in expression of *ERG24*, which encodes one of the enzymes targeted by Class II SBIs, is puzzling, since expression of *ERG11* increased in response to Class I SBIs. *ERG2* expression increased in response to Class II SBIs, but it seems odd that fungicides targeting both *ERG2* and *ERG24* would increase expression of one but not both genes. However, *ERG2* may be more responsive than *ERG24* to changes in ergosterol biosynthesis.

The ergosterol pathway gene expression levels of cells treated with the Class III SBI, fenhexamid, differed strikingly from those of cells treated with the Class I and Class II SBIs. Expression of genes from *ERG11* to *ERG4*, including the targeted *ERG27*, was essentially unchanged between fenhexamid-treated and control cells (Fig. 2C). Only *ERG10*, *ERG13*, and *ERG9* expression increased to $\geqslant 150\%$ of the control (Fig. 2C). The lack of change in gene expression was similar to the lack of change in response to cyprodinil, the MBI (Fig. 2D). Since disruption of *ERG27* has been found to cause an overall decrease in sterol production [13], the general lack of change in ergosterol pathway gene expression was surprising. However, *S. cerevisiae* is considerably less sensitive to fenhexamid than *Botrytis cinerea*, the plant pathogen used for previous fenhexamid studies [38]. While the I_{50} of fenhexamid for *B. cinerea* was 0.364 μM in liquid culture, the I_{50} for *S. cerevisiae* in this study was 280 μM [38]. Consequently, the toxicity of fenhexamid to *S. cerevisiae* may be due to some other mode of action, and ergosterol biosynthesis may not be inhibited at all. One way to determine if *ERG27* is affected by fenhexamid might be to do growth studies of yeast overexpressing *ERG27* and see if those cultures are more tolerant of fenhexamid.

3.3. Effects of fungicide treatments on methionine biosynthetic genes

As ergosterol biosynthesis was clearly not affected in MBI (cyprodinil)-treated cells (Fig. 2D), and methionine biosynthesis was expected to be affected in MBI-treated cells, expression of 13 methionine biosynthetic genes (Fig. 3) was compared in response to the MBI and the eight SBIs. MBI treatment caused three genes to be up- or down-regulated at least 2-fold ($\geqslant 200\%$ or $\leqslant 50\%$ of the control). Expression of *SAM2*, involved in the conversion of methionine into *S*-adenosylmethionine, decreased to $40 \pm 1\%$ of the control. Expression of *STR2*, thought to encode cystathionine gamma-synthase, increased to $221 \pm 13\%$ of the control. Expression of *HOM2*, which encodes aspartate semialdehyde dehydrogenase, increased to $209 \pm 35\%$ of the control. The lack of effect on *STR3* ($152 \pm 39\%$ of the control) agreed with recent findings that the cystathionine β-lyase enzyme, encoded by *STR3*, is not the primary target of cyprodinil and other anilinopyrimidines [33]. The only effect on methionine biosynthetic genes that was seen with the SBI fungicides was an in-

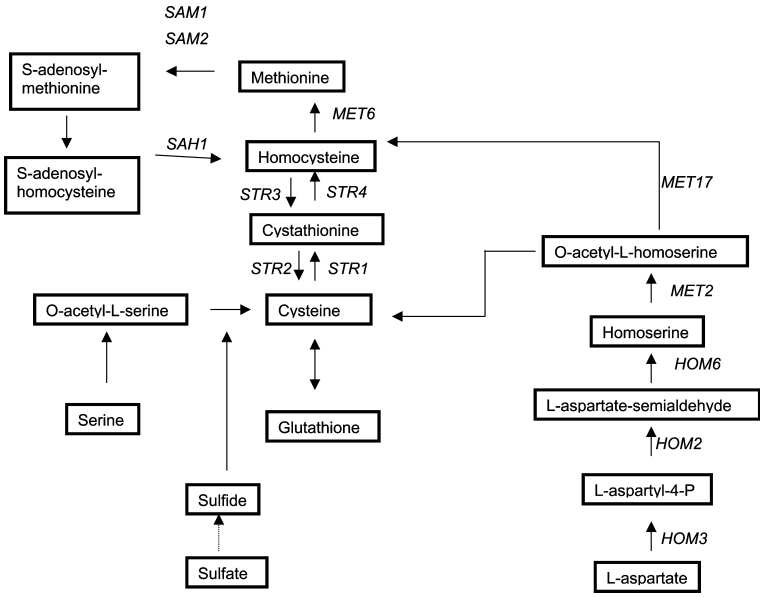

Fig. 3. Methionine biosynthetic pathway and corresponding genes, based on Fritz et al. [39] and the *Saccharomyces* genome database.

crease of 285% for *STR1* in response to fenhexamid. However, with a standard deviation of 157%, the increased *STR1* expression may not be real.

3.4. Effects of treatments on cell cycle genes

Because ergosterol and methionine biosynthesis are essential to yeast cell growth, genes involved in the cell cycle were expected to be affected by SBIs and an MBI. A large number of genes (about 800, according to Spellman et al. [40]) is involved in the cell cycle, and so a subset of 102 genes previously found to be cell cycle-controlled (http://cellcycle.www.stanford.edu/cellcycle/data/rawdata, information from [40]), was analyzed. The downregulated genes in that subset are listed in Table 4, with other downregulated genes. Upregulated cell cycle genes not listed in that subset, but found in the process of gene annotation, were included in Tables 2 and 3. No cell cycle genes were consistently upregulated by all Class I or all Class II SBIs. *RNR2* (a ribonucleotide diphosphate reductase) and *HSP12* (a heat shock protein), classified as cell cycle genes according to the Comprehensive Yeast Genome Database, were upregulated by fenhexamid in all three biological replicates (Table 2). Three other genes classified as cell cycle genes according to the Comprehensive Yeast Genome Database—the DNA-protecting glycosylase *MAG1*, the cell wall organization gene *PST1*, and the transcription initiation-related gene *TAF11*, were upregulated by fenhexamid in two of three biological replicates (Table 2). The *SED1* gene, which encodes a cell wall protein involved in stress responses, was upregulated in response to the MBI, as were the putative heat shock gene *YRO2* and the *SNZ1* gene (Table 3). *EGT2*, a hydrolase thought to be involved in cytokinesis, was downregulated (40% of the control) in response to fenhexamid and to the Class II SBI dodemorph. *CST13*, which is involved in chromosome exit from mitosis, was also downregulated in response to dodemorph. *BRN1*, involved in the condensation and segregation of chromosomes, was downregulated in response to fenhexamid (Table 4). The *BRN1* gene was not affected by treatments other than fenhexamid and hence may be a reliable marker of responses unique to Class III SBIs.

Table 2
Genes upregulated at least 2-fold ($\geqslant$200% of the control) as a result of treatment with Class I, Class II, or Class III SBIs

SBI class	Gene function	Gene	Gene description	Mean % of control ± SE	Gene also induced by class
I ($N = 5$)	Metabolism	*ERG3*	Sterol C-5 desaturase	299 ± 43	II
		ATF2	Alcohol *O*-acetyltransferase	255 ± 43	II
	Cell rescue and defense; transport; interaction with cellular environment:	*PDR5*	ABC transporter	451 ± 150 ($N = 12$)	III, MBI
II ($N = 2$)	Metabolism	*ERG3*	Sterol C-5 desaturase	357 ± 59	I
		ERG6	Sterol C-24 methyltransferase	336 ± 14	
		ATF2	Alcohol *O*-acetyltransferase	344 ± 65	I
	Metabolism; cell rescue	*ERG11*	Cytochrome P450 monooxygenase	307 ± 37	
		TSA2	Thioredoxin peroxidase	316 ± 38	
	Cell rescue	*YGP1*	Glycoprotein produced in response to nutrient limitation	475 ± 72	III, MBI
	Cellular transport	*YGR131W*	Unknown protein	262 ± 5	
III ($N = 1$)	Metabolism	*HMX1*	Heme-binding peroxidase	384 ± 183	MBI
		ICT1	Putative lipase	746 ± 247	MBI
		INO1	Inositol 3-phosphate synthase	791 ± 187	
		SFH4/PDR17	Phosphatidyl inositol transfer protein, involved in multidrug resistance	223 ± 24	
		ILV5	Reductoisomerase involved in biosynthesis of branched-chain amino acids	210 ± 19	MBI
	Metabolism; cell cycle	*RNR2*	Ribonucleotide diphosphate reductase	232 ± 22	
	Metabolism; cellular transport	*SFH3/PDR16*	Phosphatidyl inositol transfer protein, involved in multidrug resistance	474 ± 245	
		ACP1	Acyl carrier protein	266 ± 34	
	Metabolism; cell rescue	*GRE2*	Methylglyoxal reductase induced under stress	891 ± 183	MBI
	Metabolism; cell rescue; cell cycle; interaction with cellular environment	*HSP12/GLP1*	Heat shock protein, protects membranes from desiccation	864 ± 163	MBI
	Metabolism; energy; cell rescue	*ALD3*	Aldehyde dehydrogenase, involved in polyamine degradation, induced in response to stress	230 ± 40	
	Metabolism; energy	*YPL088W*	Putative alcohol dehydrogenase	351 ± 144	
	Metabolism; energy (fermentation)	*YAL061W*	Putative dehydrogenase	284 ± 81	MBI

(*continued on next page*)

Table 2 (*continued*)

SBI class	Gene function	Gene	Gene description	Mean % of control ± SE	Gene also induced by class
	Metabolism; protein fate; biogenesis of cellular components	*SRT1/YMR101C*	Prenyltransferase involved in dolichol biosynthesis	259 ± 33	
	Metabolism; protein with binding function	*YDL124W*	Amide reductase	236 ± 46	
	Cell cycle; protein with binding function; cell rescue	*MAG1*	Glycosylase protecting DNA against alkylating agents	210 ± 32	
	Cell cycle; cell type differentiation	*PST1*	Cell wall organization	216 ± 25	
	Cell cycle; transcription	*TAF11*	Involved in initiation of transcription by RNA polymerase II	228 ± 38	
	Cell rescue	*YGP1*	Glycoprotein produced in response to nutrient limitation	652 ± 315	II, MBI
		YBL064C	Mitochondrial peroxidase involved during oxidative stress	230 ± 26	
	Cell rescue; transport; interaction with cellular environment	*PDR5/LEM1*	ABC transporter	660 ± 131	I, MBI
	Cell rescue; cellular transport	*TPO1*	Polyamine transporter	486 ± 222	MBI
	Cell rescue; biogenesis of cellular components (also cell cycle; according to [40])	*SED1*	Cell wall protein; induced in response to stress; required for resistance to lytic enzymes	307 ± 98	MBI
	Cellular transport	*YOR049C/RSB1*	Membrane transporter	737 ± 247	
	Cell type differentiation	*SPS100*	Cell wall component; contributes to spore maturation	383 ± 30	
	Protein fate	*ERO1*	Involved in formation of protein disulfide bonds in ER	313 ± 73	
		YLR387C	Transcription factor (zinc finger)	256 ± 22	
		HRD2/RPN1	Proteasome subunit, involved in protein catabolism	396 ± 180	
		FPR3	May be involved in folding of ribosomal proteins	203 ± 36	
		YIM1	Mitochondrial protease	236 ± 42	
		YPS3/YPS4	Aspartic protease	211 ± 31	MBI

Protein synthesis	*YKL3/MRP8*	Ribosomal protein	330 ± 89	
Transcription	*RRN11*	Transcription initiation factor for RNA polymerase I	230 ± 46	
Energy	*OYE2*	Oxidoreductase	219 ± 22	
Classification not yet clear-cut	*RNP1*	RNA binding	304 ± 12	
Unclassified	*PIN4/MDT1*	Involved in response to DNA damage and progression from G2 to M phase in cell cycle	258 ± 21	
	TOS5	Unknown	270 ± 30	
	YAL046C	Unknown	217 ± 21	
	SHE10/YGL228W	Unknown	253 ± 36	
	YGR035C	Unknown	916 ± 146	MBI
	YGR146C	Unknown	426 ± 203	MBI
	YHR138C	Has endopeptidase inhibitor activity	370 ± 63	
	YHR209W	Putative SAM-dependent methyltransferase	318 ± 37	
	YIL041W	Unknown	248 ± 51	
	YJL171C	Unknown	230 ± 22	MBI
	YLR194C	Unknown	289 ± 81	
	YLR201C	Unknown	270 ± 52	
	YLR346C	Unknown	624 ± 180	
	YMR102C	Unknown	472 ± 83	MBI
	YNL155W	Unknown	209 ± 8	
	YOR152C	Unknown	211 ± 21	
	RSB1/YOR049C	Phospholipid-translocating ATPase	737 ± 247	MBI

Fold changes, shown as a percent of the control, were evaluated as explained in Materials and methods. Means ± standard errors (SEs) are shown for SBI classes I and II, and means ± standard deviations (SDs) are shown for SBI class III and the MBI. The number (*N*) of fungicides in each class is indicated in parentheses next to the class number.

Table 3
Genes upregulated at least 2-fold in response to cyprodinil, a putative MBI

Gene function	Gene	Gene description	Mean % of control ± SD	SBI classes inducing gene
Metabolism	*ARG10*	Argininosuccinate synthetase	481 ± 60	
	ARG3	Ornithine carbamoyltransferase	287 ± 89	
	ARG5,6	Kinase and oxidoreductase	306 ± 31	
	ARG8	Acetylornithine aminotransferase	258 ± 13	
	BAT2	Branched-chain amino acid transferase	327 ± 97	
	HAD1/BNA1	3-Hydroxyanthranilic acid dioxygenase	386 ± 38	
	HMX1	Heme-binding peroxidase, some similarity to heme oxygenases	605 ± 337	III
	ICT1	Putative lipase	530 ± 93	III
	ILV5	Reductoisomerase involved in branched-chain amino acid biosynthesis	262 ± 40	III
	YNL274C	Similar to glycerate- and formate-dehydrogenases	283 ± 50	
	BNA2	Dioxygenase required for nicotinic acid biosynthesis	355 ± 122	
Metabolism, cell cycle, cell rescue, interaction with cellular environment	*HSP12/GLP1*	Heat shock protein, protects membranes from desiccation	875 ± 177	III
Metabolism; cell rescue	*GRE2*	Methylglyoxal reductase induced under stress	224 ± 18	III
Metabolism; cellular transport	*SFH3/PDR16*	Phosphatidyl inositol transfer protein	335 ± 20	III
Metabolism; energy	*HXK1*	Hexokinase	242 ± 11	
	IDP1	Mitochondrial isocitrate dehydrogenase	344 ± 122	
	AAD10	Putative aryl alcohol dehydrogenase	222 ± 35	
	YAL061W	Putative dehydrogenase	318 ± 76	III
Metabolism; energy; biogenesis of cellular components	*ALD5*	Mitochondrial aldehyde dehydrogenase	448 ± 72	
Metabolism; protein fate	*CPS1*	Carboxypeptidase expressed under low-nitrogen conditions	242 ± 1	
Metabolism; transcription	*MAL3R/MAL33*	Maltose fermentation regulatory protein, nonfunctional in S288c	227 ± 4	
	MIG2/MLZ1	Zinc finger protein; glucose repressor	240 ± 28	
Metabolism; transcription; cellular communication	*ERN4/HAC1*	Transcription factor, essential under some stress conditions	220 ± 14	
Cell rescue	*DDR48*	DNA damage-responsive protein	350 ± 80	
	SSU1	Protein involved in sulfite transport/excretion	266 ± 25	
	SNO1	Involved in pyridoxine metabolism, expressed during stationary phase	367 ± 96	
	PST2	Flavodoxin-like protein; regulated by YAP1, a transcription factor controlling response to oxidative stress	211 ± 7	
	YGP1	Glycoprotein produced in response to nutrient limitation	619 ± 161	II, III
	YMR173W-A	Hypothetical ORF, putative transporter	296 ± 74	
Cell rescue; biogenesis of cellular components (also cell cycle, according to [40])	*YRO2*	Putative heat shock protein	301 ± 118	
	SED1	Cell wall protein; induced in response to stress; required for resistance to lytic enzymes	299 ± 35	III
Cell rescue; transport; interaction with the cellular environment	*LEM1/PDR5*	ABC transporter	856 ± 204	I, III
Cell rescue; cell cycle	*SNZ1*	Involved in pyridoxine metabolism, expressed during stationary phase	770 ± 325	

Table 3 (*continued*)

Gene function	Gene	Gene description	Mean % of control ± SD	SBI classes inducing gene
Cell rescue, cellular transport	*TPO1*	Polyamine transporter	272 ± 10	III
	TPO2	Proposed polyamine transporter	330 ± 46	
Cell rescue; transport; interaction with the cellular environment	*SNQ2*	ABC transporter involved in transport of xenobiotics	390 ± 119	
Cell rescue; protein fate; protein with binding function	*HSP26*	Heat shock protein	942 ± 32	
Cell cycle	*CLG1*	Cyclin-like protein	214 ± 12	
Cellular transport	*OMP2/POR1*	Mitochondrial porin	250 ± 6	
	YIL056W	Unknown protein, may be involved in transport facilitation	314 ± 82	
	BAP2	Amino acid transport	461 ± 28	
Cellular transport; cell rescue	*ARN1*	Ferrichrome transporter	360 ± 111	
	ARN2/TAF1	Siderophore transporter	521 ± 137	
Cellular transport; interaction with the cellular environment	*SIA1/YOR137C*	Involved in activating a membrane proton ATPase	268 ± 13	
Protein fate	*PHO9/PEP4*	Aspartyl protease	255 ± 42	
	YPS3/YPS4	Aspartic protease	273 ± 39	III
Energy, protein fate, interaction with cellular environment	*COX17*	Chaperone; involved in shuttling copper to cytochrome *c* oxidase	252 ± 10	
Interaction with cellular environment	*PRY1*	Expressed during starvation	224 ± 6	
Biogenesis of cellular components	*SPI1*	Induced during stationary phase	264 ± 2	
Transcription	*YPR015C*	Similar to known transcription factors	297 ± 78	
Classification not yet clear-cut	*YLR281C*	Some similarity to polypeptide chain release factors	246 ± 37	
Unclassified	*FIT2*	Unknown	424 ± 62	
	FIT3	Unknown	506 ± 89	
	YBL048W	Unknown	378 ± 67	
	YGR035C	Unknown	888 ± 15	III
	YGR137W	Unknown	224 ± 6	
	YGR146C	Unknown	369 ± 147	III
	YGR161C	Putative phosphatase activity	359 ± 55	
	YHR029C	Unknown	504 ± 53	
	YHR087W	Unknown	271 ± 76	
	YJL161W	Unknown	271 ± 27	
	YJL171C	Unknown	264 ± 12	III
	YKL051W	Unknown	341 ± 34	
	YMR007W	Unknown	297 ± 54	
	YMR102C	Unknown	667 ± 146	III
	YMR103C	Unknown	258 ± 65	
	YMR181C	Unknown	251 ± 29	
	YPL280W	Unknown	276 ± 9	
	YSN1	Unknown	248 ± 54	
	FIT1	Involved in iron uptake	484 ± 137	
	YBL048W	Unknown	378 ± 48	
	YJL037W	Unknown	248 ± 11	
	YOR049C	Phospholipid-translocating ATPase	808 ± 272	III

Fold changes were evaluated as explained in Materials and methods. Because only one fungicide was used, standard deviations are shown.

Table 4
Genes downregulated at least 2-fold (⩽50% of the control) following treatment with Class I, II, or III SBIs, or with cyprodinil (MBI)

Class	Gene function	Gene	Gene description	Mean % of control ± SE or SD	Other classes repressing
I ($N = 1$; prochloraz)	Interaction with the environment	*SAG1*	α-Agglutinin, promotes cell contact that facilitates mating	23.8 ± 0.2	III, dodemorph
II ($N = 1$; dodemorph)	Metabolism, interaction with the cellular environment	*RPI1*	Transcriptional regulator	37.4 ± 5.7	
	Cell cycle	*EGT2*	Hydrolase possibly involved in cytokinesis	20.7 ± 7.5	III
		CST13/CS4	Chromosome stability; involved in exiting from mitosis	27.1 ± 2.7	
	Cellular communication; interaction with the cellular environment; protein activity regulation	*STE3/DAF2*	Receptor for mating-type pheromone	30.4 ± 0.4	III
	Cell rescue; biogenesis of cellular components	*WSC4/YHC8*	Cell wall integrity, stress response	37.5 ± 1.6	
	Cellular transport, interaction with the cellular environment	*FET3*	Ferroxidase; ferrous ion transport	34.0 ± 5.4	
		FTR1	Iron transport	26.7 ± 11.7	
	Interaction with the cellular environment	*MF(ALPHA)2* (*YGL089C*)	Mating factor	19.6 ± 1.4	
	Interaction with the environment	*SAG1*	α-Agglutinin, promotes cell contact that facilitates mating	12.1 ± 0.7	Prochloraz, III
	Biogenesis of cellular components	*UTR2/CRH2*	Involved in cell wall maintenance	22.0 ± 4.5	
	Unclassified	*YDL241W*	Unknown	40.1 ± 6.1	
		YLR112W	Unknown	43.1 ± 5.5	
III ($N = 3$; fenhexamid)	Metabolism	*PYC2*	Pyruvate carboxylase	46.6 ± 6.9	
	Metabolism; energy; transcription; protein with binding function	*MIS1*	Mitochondrial C-1 tetrahydrofolate synthase	48.8 ± 9.6	
	Metabolism, protein synthesis, transcription	*FMT1*	Catalyzes formylation of initiator of protein synthesis	47.3 ± 14.7	

	Cell cycle	*BRN1*	Involved in segregation and condensation of chromosomes	47.9 ± 9.6	
		EGT2	Hydrolase possibly involved in cytokinesis	39.4 ± 19.0	Dodemorph
	Cellular communication, cell fate, cell type differentiation, biogenesis of cellular components, protein binding function	*BOI1*	Interact with various proteins including GTPases, involved in bud growth and determination of cell polarity	48.5 ± 14.9	
	Cellular communication; interaction with the cellular environment; protein activity regulation	*STE3*	Receptor for mating-type pheromone	46.3 ± 17.6	Dodemorph
	Cell fate	*BUD14*	Involved in determining location of budding site on a cell during vegetative growth	47.8 ± 13.7	
	Interaction with the environment	*SAG1*	α-Agglutinin, promotes cell contact facilitating mating	41.8 ± 9.0	Prochloraz, dodemorph
	Unclassified	*YHR095W*	Unknown	42.5 ± 12.1	
		YKL177W	Unknown	38.3 ± 6.1	
		YLR454W	Unknown	49.0 ± 11.3	
		YBR226C	Unknown	47.9 ± 6.4	
		YLR162W	Unknown	40.5 ± 9.9	
MBI	Metabolism	*FUI1*	Uridine transporter (permease)	26.8 ± 1.7	
		MET1 (MET20)	Transmethylase involved in biosynthesis of siroheme	41.7 ± 1.3	
		SAM2	*S*-adenosylmethionine synthetase	39.9 ± 1.6	
		BIO2	Biotin synthase	39.5 ± 4.6	
	Cell rescue	*YHB1*	Flavohemoglobin, may be involved in stress responses	42.3 ± 3.5	
	Cellular transport	*HNM1*	Choline transporter (permease)	44.1 ± 1.6	
	Unclassified	*TOS4*	Transcription factor that binds to many promoter regions, including some for cell cycle genes	41.4 ± 0.05	
		YMR215W	Putative glucosyltransferase	46.3 ± 0.01	

As no genes were downregulated by fenpropimorph, only gene changes for dodemorph are shown for Class II SBIs. Means ± SDs are shown.

3.5. Effects of treatments on branched-chain amino acid biosynthetic genes

To evaluate the effects of fungicide treatment on a pathway that should not have been directly affected by ergosterol biosynthesis inhibitors, expression of the genes involved in the biosynthesis of branched-chain amino acids (valine, leucine, and isoleucine) was studied. Eleven genes involved in those pathways (http://pathway.yeastgenome.org) were examined. The only SBI treatment to induce a significant change was fenhexamid, which increased *ILV5* expression to $210 \pm 19\%$ of the control. Since *ILV5* also has a role in maintaining the stability of mitochondrial DNA [41], the increase in *ILV5* expression may indicate that fenhexamid affects respiration. Three genes were upregulated by more than 200% of the control in response to cyprodinil (*ILV5*, $262 \pm 40\%$; *ILV6*, $220 \pm 0.7\%$; *BAT2*, $327 \pm 98\%$). This increase in gene expression could indicate that cyprodinil affects branched-chain amino acid metabolism as well as methionine biosynthesis, or it could indicate a slowdown in primary metabolism due to cell stress, and an increase in biosynthetic enzyme activity in response to the resulting depletion of amino acids.

3.6. Effects on other genes

No gene was upregulated 2-fold or more by all five Class I inhibitors used in this study (Table 2). However, *ERG3* and *ATF2* were upregulated by all Class I inhibitors except for prochloraz, and *PDR5* was upregulated by all Class I inhibitors except for fenbuconazole. The *PDR5* gene encodes an ATP-binding cassette implicated in efflux of toxic compounds [42]; thus, its upregulation is a logical cellular response to fungicide treatment. Since *PDR5* was also upregulated in response to fenhexamid (Table 2) and to the MBI (Table 3), and to steroids in another study [43], induction of this gene may be a standard response to xenobiotics. *ATF2*, an alcohol acetyltransferase, is implicated in *PDR5*-mediated steroid detoxification [44]. Since it was upregulated by Class II SBIs in this study (Table 2) and is upregulated by ketoconazole, a pharmaceutical Class I SBI [21], *ATF2* may work with *PDR5* in efflux of SBIs as well as steroids.

Seven genes were induced by both Class II inhibitors used in this study (Table 2). Besides the three ERG genes listed (a smaller subset than shown in Fig. 2, because only genes whose expression increased at least 2-fold in both replicates were considered), *ATF2* was induced, indicating that this response may be a general response to SBIs. The other upregulated genes had roles in responses to nutrient limitation (*YGP1*), cell growth and possibly oxidative stress (*TSA2* [45]); and transport (*YGR131W*). Induction of *YGP1* may be another general response to stress, as it was also induced by fenhexamid and cyprodinil (Tables 2 and 3).

Fifty-one genes responded to fenhexamid with at least a 2-fold increase in expression in two of three biological replicates (Table 2). The high number (relative to Class I and Class II) of genes responsive to a Class III inhibitor may indicate that reproducibility among biological replicates is greater within one fungicide than within multiple fungicides, even if the multiple fungicides have the same target site. No ergosterol biosynthetic genes were up- or downregulated at least 2-fold, thus supporting the results of Fig. 2C. Thirteen of the responsive genes had unknown functions. Some genes involved in lipid metabolism were upregulated (e.g., the acyl carrier protein gene *ACP1*, the putative lipase gene *ICT1*, and the phosphatidyl inositol transfer genes *SFH3* and *SFH4*). Since other lipids besides ergosterol are present in fungal membranes, disrupting ergosterol production may affect other membrane components as well. Some of the genes encoded functions that could be necessary to restore homeostasis after cellular perturbation: three genes (*YPL088W*, *YAL061W*, and *OYE2*) had functions related to respiration; six were involved in the cell cycle (*RNR2*, *MAG1*, *PST1*, *TAF11*, *SED1*, and *HSP12*); and one (*YKL3*) was involved in protein synthesis. The upregulation of genes involved in protein fate (*ERO1*, involved in protein disulfide bond formation [46]; *HRD2*, a proteasome subunit; *FPR3*, which may be involved in protein folding; *YIM1* and *YPS3*, both proteases; and *YLR387C*, a transcription factor) may indicate that fenhexamid

treatment causes degradation or modification of existing proteins and transcription leading to new proteins. The upregulation of *INO1*, which encodes inositol 3-phosphate synthase, as well as *SFH3* and *SFH4*, suggests that cell response to fenhexamid includes biosynthesis of signaling molecules. The response also includes upregulation of genes involved in cell rescue (*GRE2*, *TPO1*, *PDR5*, *ALD3*, *MAG1*, *YGP1*, *YBL064C*, and *HSP12*). Except for *PDR5* and *YGP1* (a glycoprotein produced in response to nutrient limitation), the genes upregulated by fenhexamid treatment were different from those upregulated by Class I or Class II inhibitors, reinforcing the evidence from ergosterol biosynthetic genes (Fig. 2C) that fenhexamid treatment results in an expression fingerprint very different from that of Class I and Class II inhibitors. Sixteen (31%) of the 51 fenhexamid-induced genes were also induced by the MBI (Tables 2 and 3). Those genes were *YGP1*, *PDR5*, *HMX1* (a peroxidase), *ICT1*, *GRE2*, *HSP12*, *YGP1*, *TPO1*, *SED1*, *ILV5*, *YAL061W* (a putative dehydrogenase), and the unknown genes *YGR035C*, *YGR146C*, *YJL171C*, *YMR102C*, and *RSB1 (YOR049C)*. Fenhexamid's profile does differ from those of the other two classes of SBIs, but since only 16 of cyprodinil's 73 induced genes were induced by SBI treatment (22% of the MBI-induced genes), fenhexamid's profile is also distinct from that of an MBI.

Few genes were significantly downregulated by the treatments used in this study (Table 4). In response to Class I SBIs, the *SAG1* gene, an agglutinin that facilitates mating, was repressed only by prochloraz ($23.8 \pm 0.2\%$ of the control). In response to the Class III SBI, *SAG1* was also repressed, along with the cell cycle genes *EGT2* and *BRN1*, five genes of unknown function, a tetrahydrofolate synthase-encoding *MIS1*, *FMT1* (involved in protein synthesis initiation), *BOI1* (involved in bud growth), *STE3* (pheromone receptor) and a pyruvate carboxylase-encoding *PYC2*. No genes were significantly downregulated by fenpropimorph, although dodemorph, the other Class II SBI, repressed twelve genes, including *SAG1* and *EGT2*. None of the genes downregulated by the SBI treatments were downregulated by the MBI.

In summary, treatment of *S. cerevisiae* cultures with different classes of ergosterol biosynthesis inhibitors, at I_{50} concentrations, resulted in gene expression patterns that included general xenobiotic or stress responses, and some responses that may be unique to the class of SBI used. General responses included upregulation of transporters and proteins produced in response to nutrient limitation, upregulation of genes involved in respiration, and (in a small subset of treatments) downregulation of genes involved in mating and cell division. The *ERG3* gene was upregulated in response to Class I and Class II inhibitors, in agreement with other microarray studies. *ERG6* and *ERG11*, which have been found to respond to Class I pharmaceutical SBIs, were upregulated in response to both Class I and Class II SBIs in this study. More changes in *ERG* gene expression were seen in response to Class II SBIs. The pattern of expression of ergosterol biosynthetic genes was similar in response to Class I and Class II SBIs, but very different in response to the Class III SBI, which did not cause any significant responses in those genes. This lack of change in ergosterol biosynthetic gene expression may indicate that the toxicity of fenhexamid to *S. cerevisiae* is due to another mode of action.

Given the small number of ergosterol biosynthetic genes that responded significantly to SBIs in this study, the list of up- or downregulated genes (Tables 2–4) would not alone suffice to identify ergosterol biosynthesis inhibition as the SBI mode of action. However, analyzing expression of genes in a biosynthetic pathway, as done in Fig. 2, points to a pattern in which ergosterol biosynthetic gene expression appears affected above background levels by Class I and II inhibitors. With a single fungicide, consistent upregulation of two to four genes, out of a pathway encoded by 23 genes, is probably not random. Furthermore, the reasonably similar gene responses to all of the Class I and II inhibitors is highly unlikely to be random. Similarly, the change in expression of two out of thirteen methionine biosynthetic genes in response to cyprodinil is probably a reliable indication that this compound indeed affects methionine biosynthesis. Also, the lack of up- or downregulation of genes in the ergosterol pathway, as seen in the Class III SBI and the MBI, is probably a reliable indication that these compounds do not affect

ergosterol biosynthesis in yeast. These results suggest that when searching for expression fingerprints in response to unknown fungicides, looking for gene expression patterns within different pathways may help to identify a mode of action, thus providing—as microarrays should provide—a first step towards determining the mode of action of unknowns by work with mutants or real-time PCR.

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